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13. ABSTRACT ***(Maximum 200 Words)***

Heregulin (HRG) is a growth factor that activates *erbB-2-3-4* receptors. We have generated a novel model of tumor progression from a hormone-dependent to a hormone-independent phenotype by introducing HRG into breast cancer cells. We now would like to investigate the mechanism by which HRG induces tumor progression. Our working hypothesis is that expression of HRG induces an uncontrolled mitogen-activated protein kinase (MAPK) cascade producing unbalanced growth promoting genes. **The proposed studies aim to determine whether blocking MAPK activation, cells revert to become hormone-dependent and antiestrogen responsive.**

During the first year of funding, we maintained the timeline outlined in the statement of work: a) Generated a *Ras* dominant negative (N17) regulated expression vector; b) Performed transfections into a number of MCF-7/HRG cells and isolated specific drug-resistant clones; c) Partially characterized these clones; and d) Initiated the construction of the MAPK mutant. The major findings of our work are that expression of N17 in MCF-7HRG cells results in reversion from an anchorage-independent to anchorage-dependent phenotype. Moreover, when analyzing the response to estradiol, MCF-7/HRG/N17 cells regained hormonal response to a level of the wild type MCF-7 cells. **This data demonstrates activation of the MAPK via the HRG pathway promotes an aggressive hormone-independent phenotype.**

14. SUBJECT TERMS
Breast Cancer, Heregulin, Estrogen Receptor, MAPK, Ras, Anchorage-Independent, Antiestrogen resistance, FTS.

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4. TABLE OF CONTENTS

5. INTRODUCTION

Clinical studies have shown that the *erbB-2* oncogene product, when overexpressed, correlates with tamoxifen resistance in estrogen receptor positive (ER+) breast cancer specimens. The response rate to tamoxifen in the metastatic setting varies from 50-75% in ER positive patients. In patients whose tumors overexpress *erbB-2* in the context of the ER receptor, this response rate decreases to 17%. Although the presence of ER is employed to predict the hormone dependency of a tumor, the relationship with response to endocrine therapy is not absolute (not all ER+ patients respond to endocrine therapy). Significant levels of ER have been detected in over 60% of human breast cancers, but at best only two-thirds of these ER positive tumors respond to endocrine therapy. Why this should occur is unclear. However, our experimental studies have demonstrated a relationship between the ER and *erbB-2* signaling pathways. For example, it has been shown that estradiol down regulates *erbB-2* in overexpressing cells and that ER is required for this to occur. Potentiation of breast cancer cell growth by either the ER or *erbB*-pathway may make cells less amenable to anti-proliferative strategies directed to the alternative pathway.

It has been shown that inhibition of Ras-dependent signaling and the oncogenic function of Ras by farnesyl transferase inhibitors and/or blockers of Ras membrane anchorage are limited due to alternative prenisylation of Ras. It has been shown that inhibition of the Ras-dependent Raf1-MAPK cascade is activated by S-farnesylthiosalicicyclic acid, which affects Ras membrane association but not Ras farnesylation. These data and our preliminary studies lead us to further hypothesize that *ras* activity contributes to the acquisition of an aggressive hormone-independent breast cancer phenotype. To assess this hypothesis, we employed a compound that inhibits ras anchorage to the membrane named FTS. This reagent represents a new class of Ras antagonists that may be useful for the inhibition of various types of oncogenic Ras isoforms independently of their prenylation.

We have generated a unique breast cancer tumor progression model and are equipped to design and evaluate ways to revert the progressive phenotype. We showed that MCF-7 cells, which are ER-positive, progressed to a more aggressive phenotype and rendered tumorigenic and metastatic *in vivo* merely by transfecting them with HRG. It may therefore be possible to inhibit both the uncontrolled cell proliferation and the metastatic properties of breast cancer cells by blocking either HRG or the MAPK pathway. We predict that cells can bypass their normal estrogenic requirements, *if* they develop an alternative escape mechanism. One alternative pathway appears to be the *erbB*-receptors pathway. We hypothesize that there is compensation between the alternative signaling pathways. Thus, blocking one receptor pathway will result is the re-activation of the other. Clinically, that is in patients that are positive for both ER and *erbB-*, treatment with tamoxifen may result in increased proliferation through the *erbB-* signal transduction pathway. Conversely, interrupting the *erbB-* growth pathway with signaling

blockers, ligand blockers or by other mechanisms may enhance proliferation through the ER system, and thereby restoring antiestrogen sensitivity.

To assess the mechanism by which HRG promotes this aggressive phenotype, we first determined the involvement of the *ras*-signaling cascade in this process. We observed an increased in activated *ras* in the HRG transfected MCF-7 cells (MCF-7/HRG) when compared to the control MCF-7 cells. Furthermore, a marked increase in activation of mitogen-activated protein kinase (MAPKP) was seen in the MCF-7/HRG cells.

The proposed studies will provide a better understanding of the pathway by which cells acquire a hormone-independent phenotype and will help us to design targeted therapies for this particular population of breast cancer patients.

6. BODY

The goal of the research outlined in this proposal is to extend ongoing studies. The following experiments are designed to shed light on the biological and molecular mechanisms by which HRG mediates and/or induces cellular transformation of breast cancer cells to a more invasive and hormone-independent phenotype. The original technical objectives were as follows:

Task 1: **To determine whether changes upstream of MAPK activity play a role in HRG induction of hormone-independent breast cancer phenotype.** We will determine if Ras activation is necessary or sufficient to block HRG action. These studies will be accomplished by transfecting a dominant negative Ras mutant (N17) in a Tet-regulated expression vector into MCF-7/HRG cells. Following the transfection, drug resistant clones will be isolated and characterized. This characterization includes determining the ability of these cells to become anchorage- as well as hormone-dependent.

Task 2: **To determine the direct effect of MAPK activity, using dominant-negative MAPK mutants.** These studies will be accomplished by transfecting a dominant negative MAPK mutant in a Tet-regulated expression vector into MCF-7/HRG cells. Following the transfection, drug resistant clones will be isolated and characterized. Characterization includes determining the ability of these cells to become anchorage-dependent as well as hormone-dependent.

Task 3: **To determine the ability of specific pharmacological kinase inhibitors (PD98059-MEK1 inhibitor and PD158780- HRG/*erbB*- inhibitor) to restore hormonal responsiveness of the HRG transfected cells.** These studies will be performed *in vitro* using MCF-7/HRG cells. Other inhibitors could be used as they become available.

STATEMENT OF WORK:

ACCOMPLISHED OBJECTIVES FROM TASKS 1-3:

STATEMENT OF WORK

Task 1: Months 8-16: Biochemical and biological characterization of isolated clones: Anchorage-dependent and -independent growth assays. Assays will be performed in the presence or absence of estradiol. Biochemical characterization: measurements of *erbB*-receptor tyrosine phosphorylation and MAPK and *Ras* activity. To begin the *in vivo* experiments.

BIOLOGICAL CHARACTERIZATION OF THE N17 EXPRESSING CELLS *IN VITRO* USING ANCHORAGE-DEPENDENT AND -INDEPENDENT GROWTH ASSAYS:

We determined that MCF-7/HRG cells grow in an anchorage-independent fashion in the absence of estradiol. In contrast, the control cells (MCF-7/V1) grow exclusively in the presence of estradiol. Analysis of the N17-transfected MCF-7/HRG cells (MCF-7/HRG/N17) resulted in inhibition of anchorage-independent growth, as compared with control MCF-7/HRG cells. Moreover, when analyzing the response to estradiol, MCF-7/HRG/N17 cells regained the hormonal response to a level typical of the wild type MCF-7 cells. The ability of the dominant negative Ras mutant to abolish HRG induction of the anti-estrogen resistant phenotype will confirm the requirement of Ras in the induction of a Tam-resistant phenotype. **We have recently isolated a series of clones from cells that were transfected with the N17 regulated promoter vector. These cells are currently been characterized biologically *in vitro* and future studies will be performed *in vivo*.**

Task 2: Months 12-16: Biochemical characterization: measurements of *erbB*-receptor tyrosine phosphorylation and MAPK and *Ras* activity.

Task 3: Months 20-28: To characterize the biological activity of the specific pharmacological agents in vitro using all of the transfected and control cell lines: Measurements of erbB-receptor tyrosine phosphorylation, MAPK activity. To biologically characterize the kinase inhibitors: Anchorage-dependent and -independent growth assays. Assays will be performed in the presence or absence of estradiol. To continue with the biological characterization of transfected cells in vivo.

EFFECT OF FTS ON MAPK ACTIVITY OF MCF-7/HRG CELLS

We first examined whether FTS can affect MAPK activity and expression in cells that have been transfected with HRG and have higher levels of MAPK activity. Since a marked increased in MAPK phosphorylation was observed in MCF-7/HRG cells, if was interesting to determine whether FTS can block MAPK activity. MCF-7/HRG cells were treated with increasing concentrations of FTS. As shown in Figure 1, our results demonstrate that FTS treatment blocks MAPK-activity and does not have any effect on expression of MAPK. Demonstrating that FTS is a specific compounds which blocks activation of the Ras-signaling pathway. We are currently in the process of assessing the effect of FTS on the phosphorylation of the erbB- receptors.

EFFECT OF FTS ON THE GROWTH OF MCF-7/HRG CELLS

Studies were performed to assess the effects of FTS on breast cancer cell growth. Heregulin expressing and non-expressing cell lines were treated with increasing concentrations of FTS. As can be seen in Figure 2, a marked growth inhibition was observed in the heregulin expressing cells (T6).

Task 2: **To verify the direct effect of MAPK activity, using dominant-negative MAPK mutants (ΔMAPK)**

Months 10-16: Cloning the dominant negative ΔMAPK mutant into an expression vector. Transfection of a dominant negative MAPK mutant (ΔMAPK), cloning of single clones expressing ΔMAPK regulated by Tetracycline.

CLONING THE ΔMAPK cDNA INTO AN EXPRESSION VECTOR: During the course of the last year we have generated the expression vectors. We have introduced into the cells the vector expressing the tetracycline promoter. After confirming the chosen expression system was successfully expressed we are in the position to transfect the ΔMAPK mutant into the Tet-regulated expression vector (Invitrogen). Insert-less vectors will be transfected as controls.

TASKS REMAINING TO BE PERFORMED FROM INITIAL TASKS 1 AND 2:

Task 1: **To determine whether changes upstream of MAPK activity play a role in HRG induction of hormone-independent breast cancer phenotype.**

Months 16-26: To continue the Characterization of the N17 transfected cells *in vitro and in vivo*: animal experiments will be performed in the presence or absence of estradiol and antiestrogens Tamoxifen and ICI 182,780.

Task 2: To verify the direct effect of MAPK activity, using dominant-negative MAPK mutants (ΔMAPK)

Months 16-22: Continue the transfection of a dominant negative MAPK mutant (ΔMAPK). Cloning of single clones expressing ΔMAPK regulated by Tetracycline.

Task 3: To determine the ability of specific pharmacological kinase inhibitors (PD98059-MEK1 inhibitor and PD158780- HRG/*erbB*- inhibitor) to restore hormonal responsiveness of the HRG transfected cells. These studies will be performed *in vitro* using MCF-7/HRG cells.

Months 20-28: To characterize the biological activity of the specific pharmacological agents in vitro using all of the transfected and control cell lines using kinase inhibitors.

TASKS REMAINING TO BE PERFORMED DURING THE NEXT YEAR FROM INITIAL STATEMENT OF WORK

Task 1: To determine if changes upstream of MAPK activity play a role in HRG induction of hormone-independent breast cancer phenotype by transfecting a dominant negative Ras mutant (N17) in a Tet-regulated expression vector into MCF-7/HRG cells and determine the ability of the N17-transfected cells to regain hormonal response;

Months 16-26: To continue the characterization of the N17 transfected cells in *vivo*: animal experiments will be performed in the presence or absence of estradiol, tamoxifen and IC1 182,780

Task 2: To verify the direct effect of MAPK activity, using dominant-negative MAPK mutants (ΔMAPK)

Months 16-22: Continue with the biochemical and biological characterization of isolated clones. Biochemical characterization: Measurements of *erbB*-receptor tyrosine phosphorylation, MAPK activity. Biological characterization: Anchorage-dependent and -independent growth assays. Assays will be performed in the presence or absence of estradiol. To continue with the biological characterization of transfected cells *in vivo*.

Months 22-36: To continue the characterization of the ΔMAPK transfected cells *in vivo*: animal experiments will be performed in the presence or absence of estradiol, tamoxifen and IC1 182,780

Task 3: To determine the ability of specific pharmacological kinase inhibitors (PD98059-MEK1 inhibitor and PD158780- HRG/*erbB*- inhibitor) to restore hormonal responsiveness of the HRG transfected cells.

Months 20-28: To characterize the biological activity of the specific pharmacological agents in vitro using all of the transfected and control cell lines using *PD98059-MEK1 inhibitor and PD158780- HRG/erbB*- inhibitors.

7. KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that a Ras blocker (FTS) can indeed block MAPK activity in the HRG expressing cells.
- Demonstrated that a Ras blocker (FTS) can inhibit the growth of the HRG expressing cells.
- The dominant negative Ras mutant was successfully transfected into breast cancer cells that overexpress HRG using a regulated promoter.
- Isolated many individual regulatable N17 expressing cells.
- Demonstrated expression of the dominant negative mutant by RNAse protection assays.
- Demonstrated that blockage of Ras activation reverts HRG expressing cells from estrogen-independent to -dependent, therefore protecting breast cancer cells from becoming hormone-independent.
- Introduced the dominant negative ΔMAPK mutant into an expression vector that can be regulated.

8. REPORTABLE OUTCOMES

- Development of cell lines.
- Development of animal models.
- A ras inhibitor can specifically block the growth of HRG expressing MCF-7 cells.

9. CONCLUSIONS

The inverse correlation between HRG and ER expression in breast cancer cell lines prompts us to hypothesize that HRG triggers a cascade of events that lead to a hormone-independent phenotype. Therefore, we transfected HRG cDNA into an ER-positive breast cancer cell line MCF-7. The first observation after transfection was that the *erbB*- receptor signaling pathway appears constitutively activated, as shown by receptor phosphorylation and higher basal levels of MAPK activity. In addition, under estrogen-deprived conditions, the doubling time for the MCF-7/HRG cells was significantly shorter than the control cells (MCF-7/wild type or MCF-7/Vector). Moreover, estradiol (E_2) did not induce the proliferation of MCF-7/HRG cells, in contrast to control cells that were clearly stimulated. Interestingly, the MCF-7/HRG cells were unresponsive to estradiol in an anchorage-dependent fashion and also grew in an anchorage-independent fashion in the absence of Estradiol. In contrast, the control MCF-7 cells were totally dependent upon estradiol stimulation for anchorage-independent growth. MCF-7/HRG cells were hormone-independent and anti-estrogen resistant *in vivo*. The tumors developed by MCF-7/HRG cells in the presence of Tam were larger than those developed in its absence. Control cells (MCF-7/V) did not form tumors in the absence of E_2 and were tumorigenic exclusively in the presence of estradiol and sensitive to Tam, as predicted.

To decide whether the acquisition of the hormone-independent phenotype was a result of the loss of ER expression and/or ER function, we determined both the level of ER expression and the modulation of progesterone receptor (PgR) expression by estradiol. The level of ER in MCF-7/HRG was lower than the control cells and, more importantly, E_2 did not regulate the level of PgR expression. Our initial results imply that the HRG-transfected cells have lost ER function. Thus it seems that constitutive expression of HRG not only down-regulates ER but also blocks its E_2-mediated activation, abolishing the induction of PgR by $E_{estradiol}$

We hypothesized that increased activation of the *Ras-Raf-MAPK* pathway by HRG would result in transactivation of the ER receptor, thereby losing ER function. Therefore, it may be that blocking these signaling cascade cells will revert the hormone-independent phenotype induced by HRG. To validate our hypothesis we generated a *Ras* dominant negative (N17) expression vector and performed transfection. We have already determined that MCF-7/HRG cells grow in an anchorage-independent fashion in the absence of estradiol. In contrast, the control cells (MCF-7/V1) grow exclusively in the presence of estradiol. Analysis of the pool population of the

N17-transfected MCF-7/HRG cells (MCF-7/HRG/N17) resulted in inhibition of anchorage-independent growth, as contrasted with control MCF-7/HRG cells. Moreover, when analyzing the response to estradiol, MCF-7/HRG/N17 cells regained the hormonal response to a level typical of the wild type MCF-7 cells.

Our preliminary studies have clearly demonstrated a mechanism by which HRG-mediated ER deactivation. We have demonstrated the biological significance of blocking the MAPK pathway in restoring estrogen responsiveness. By have done this by blockage of the activated ras using a dominant-negative mutant and by the blockage of Ras anchorage to the membrane using a compound FTS. We have clearly shown using two independent approaches that Ras and MAPK activation are key mediators of breast tumor progression from a hormone-dependent to a hormone-independent phenotype. Our future studies are aimed at defining the function of these important signal transducers in breast cancer cells, especially how they control and modulate responses to growth-inducing factors in vivo. Interference with the action of specific MAPKs will provide new intervention strategies to halt breast cancer progression.

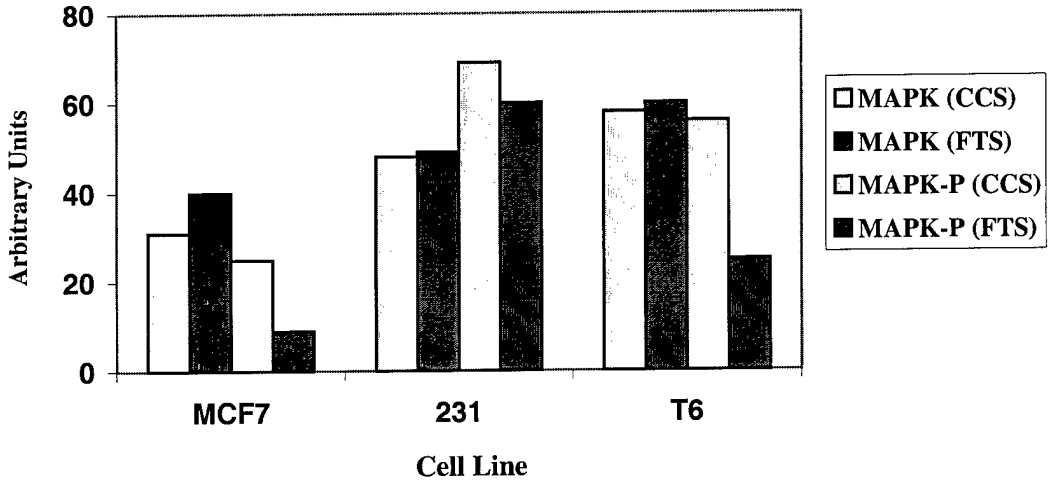
10. REFERENCES

Not Applicable

11. APPENDICES

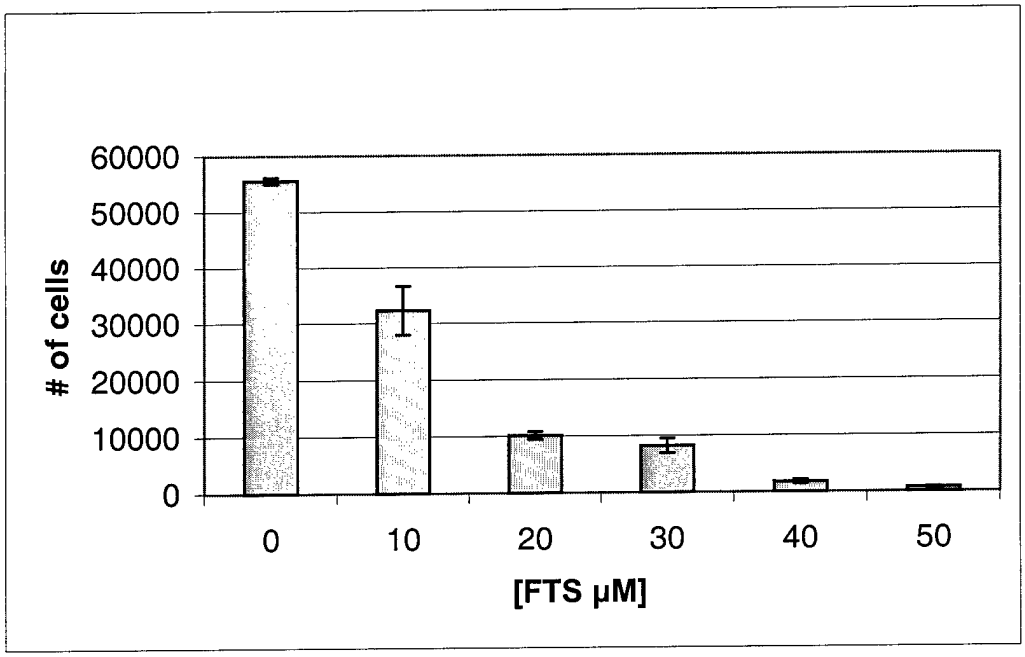
Appendix I: Figures and Figure legends

Figure 1. Effect of FTS on MAPK activity



MCF7, MDA-MB-231, and T6 cells were treated for 24hrs. with FTS. Cells were lysed and the proteins were extracted from the cell lysates. Equal protein concentrations were loaded onto an SDS-PAGE gel electrophoresis. Western blots were incubated with MAPK and phospho specific MAPK (MAPK-P) antibodies. The above results are obtained from densitometry analysis of the western blots.

Figure 2: Effect of increasing concentrations of FTS on the growth of MCF-7/HRG transfected cells.



MCF7/HRG (T6) cells were cultured in IMEM and treated with increasing concentrations of FTS for 5 days. On day 5, cells were counted n a Coulter Counter. The results represent the number of cells in the different treatments. Results were performed in triplicates.